Chloride Transport Across Rat Ileal Basolateral Membrane Vesicles (43504)

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Abstract. **The present study was designed to investigate Cl^- transport across rat ileal basolateral membranes. Basolateral membrane vesicles were prepared by a well-validated technique. The purity of the basolateral membrane vesicles was verified by marker enzyme studies and by studies of d-glucose and calcium uptake. Cl^- uptake was studied by a rapid filtration technique. Neither an outwardly directed pH gradient, nor a HCO_3^- gradient, or their combination could elicit any stimulation of Cl^- transport when compared with no gradient. 4,4-Diisothiocyanostilbene-2,2-disulfonic acid at 5 m*M* concentration did not inhibit Cl^- uptake under gradient condition. Similarly, the presence of the combination of outwardly directed Na^+ and HCO_3^- gradients did not stimulate Cl^- uptake compared with the combination of K^+ and HCO_3^- gradients or no HCO_3^- gradient. This is in contrast to our results in the brush border membranes, where an outwardly directed pH gradient caused an increase in Cl^- uptake. Cl^- uptake was stimulated in the presence of combined Na^+ and K^+ gradient. Bumetanide at 0.1 m*M* concentration inhibited the initial rate of Cl^- uptake in the presence of combined Na^+ and K^+ gradients. Kinetic studies of bumetanide-sensitive Cl^- uptake showed a V_{max} of 5.6 ± 0.7 nmol/mg protein/5 sec and a K_m of 30 ± 8.7 m*M*. Cl^- uptake was stimulated by an inside positive membrane potential induced by the ionophore valinomycin in the setting of inwardly directed K^+ gradient compared with voltage clamp condition. These studies demonstrate two processes for Cl^- transport across the rat ileal basolateral membrane: one is driven by an electrogenic diffusive process and the second is a bumetanide-sensitive $Na^+/K^+/2\ Cl^-$ process. Cl^- uptake is not enhanced by pH gradient, HCO_3^- gradient, their combination, or outwardly directed HCO_3^- and Na^+ gradients.** [P.S.E.B.M. 1992, Vol 201]

Studies utilizing short-circuited intestinal segments in several animal species (1–5) have shown that Cl^- was transported across the mucosa via a carrier-mediated process. These studies have shown that the neutral Na^+ and Cl^- coupled process was the result of the parallel operation of two transport proteins mediating the exchange of Na^+ with H^+ and Cl^- with HCO_3^-. These two processes occur at the brush border membrane of the intestine and participate in the movement of Na^+, Cl^-, and water to the body.

The processes involved in Cl^- transport across the basolateral membrane of the enterocytes are not known. Since there appears to be an electrochemical gradient favorable for Cl^- movement from the cell into the serosal fluid, a downhill process along that gradient was suggested (6, 7). However, there is no direct study to confirm this process. The possibility of a double exchange Cl^--HCO_3^- or anion cannot be ruled out. The findings of Cl^-/HCO_3^- process at the basolateral membrane of the amphiuma support the possibilities (8). This Cl^-/HCO_3^- process is inhibitable by stilbene derivatives (3). Therefore, the present studies were designed to investigate Cl^- transport across rat ileal basolateral membranes.

Materials and Methods

Preparation of Basolateral Membrane Vesicles. Adult Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). Basolateral membrane vesicles were prepared using a modified centrifugation technique followed by separation on a Percoll density gradient (9, 10), which we describe in detail elsewhere

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(10). Briefly, the distal one third of intestine was filled with citrate buffer incubated at 37°C for 15 min. The intestine was then emptied and refilled with ice-cold buffer containing 100 m*M* mannitol, 100 m*M* KCL, and 24 m*M* HEPES-Tris (pH 7.4), and gently palpated for 5 min to release the epithelial cells. The contents were then drained into a beaker on ice and the volume brought up to 250 ml in the same buffer. The cells were centrifuged at 200*g* for 5 min. The pellet was then homogenized using an Omni-Mixer (Waring Products, New Hartford, CT) for 3 min. The homogenate was then centrifuged at 2,500*g* for 20 min. The supernatant was collected and centrifuged at 22,000*g* for 25 min. The resulting fluffy layer of the pellet was resuspended in 90 ml of the same buffer and homogenized in a glass Teflon homogenizer (20 strokes). The resultant homogenate was mixed with Percoll (Pharmacia, Uppsala, Sweden) at a concentration of 15.4% and centrifuged at 48,000*g* for 45 min. A distinct band of basolateral membrane was seen at the upper one third of Percoll gradient. The band was aspirated and diluted in 100 m*M* mannitol, 100 m*M* KCl, and 24 m*M* HEPES/Tris (pH 7.4). The diluted band was then centrifuged at 48,000*g* for 20 min. The pellet was resuspended in appropriate transport buffer, then centrifuged at 48,000*g* for 20 min twice, and finally resuspended with a syringe and a 25-gauge needle in a desired volume of preincubation buffer, as described in the figure legends.

$H^{36}Cl$ (6.5–13.8 mCi/gm of Cl^-) was obtained from ICN (Irvine, CA). All other reagents were obtained from Sigma Chemical (St. Louis, MO) and were of the highest purity available.

Biochemical and Functional Purity of Vesicles. The purity of basolateral membrane preparations was determined by marker enzyme activity. Leucine aminopeptidase activity was measured by a kit from Sigma (No. 251). Na^+-K^+-ATPase activity was measured by the method of Scharschmidt *et al.* (11). Cytochrome *c* oxidase and NADPH-cytochrome *c* reductase activity were determined as described by Beaufay *et al.* (12). D-Glucose and calcium uptake was studied as described previously by our laboratory (10).

Transport Measurements. Uptake of radiolabeled ^{36}Cl by basolateral membrane vesicles was measured by a rapid filtration technique (13). Vesicles were preincubated for 2 hr at room temperature and gassed with either 95% N_2 and 5% CO_2 or 100% N_2, depending upon whether uptake was with or without HCO_3^-. Transport was initiated by adding 20 μl of vesicles to 80 μl of incubation medium at room temperature. Intravesicular and extravesicular osmolarity were maintained. At the end of the desired time interval, uptake was stopped by the addition of 2 ml of ice-cold stop solution containing 150 m*M* potassium gluconate, 8 m*M* Tris, and 18 m*M* HEPES (pH 7.5). The stop solution containing the vesicles was immediately pipet-

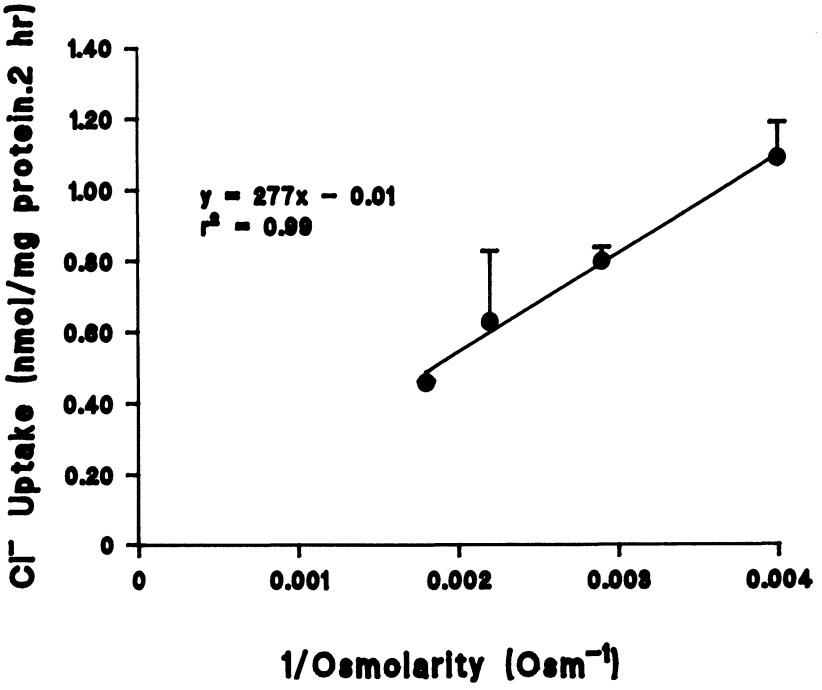

Figure 1. Effect of media osmolarity on Cl^- uptake. Vesicles were preincubated in 50 m*M* $kHCO_3^-$, 35 m*M* Tris, 40 m*M* potassium gluconate, and 75 m*M* mannitol (pH 7.5), and were gassed with 95% N_2 and 5% Co_2. Cl^- uptake was measured at 2 hr in a solution containing 0.285 m*M* $kHCO_3^-$, 6 m*M* Tris, 6 m*M* HEPES, 58 m*M* morpholino-ethane-sulfonic acid, 40 m*M* potassium gluconate, 2.1 m*M* gluconic acid (pH 5.2), and a variable amount of mannitol to adjust the final osmolarity between 250 and 600 mOsm. The relationship between 1/osmolarity and Cl^- uptake was linear ($r^2 = 0.99$), with an intercept close to zero, which indicated that Cl^- uptake represents a transmembrane phenomena.

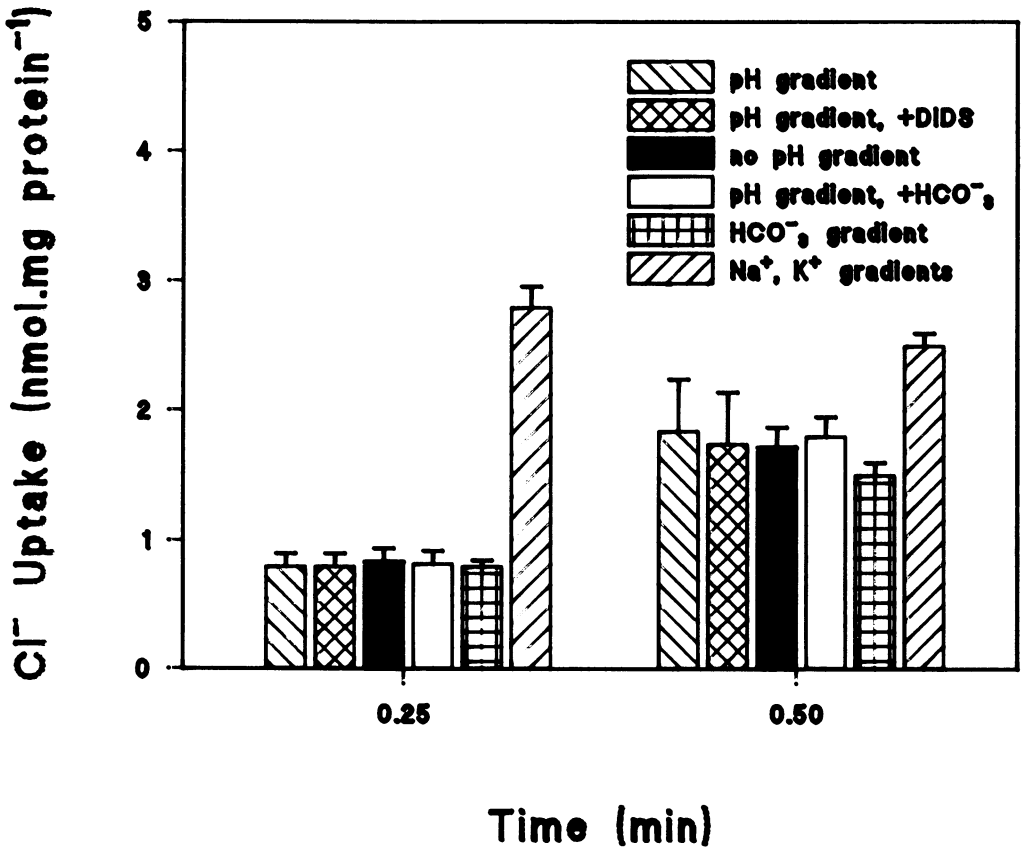

Figure 2. Characterization of Cl^- uptake. Vesicles were preincubated at pH 7.5 either with HCO_3^- (50 m*M* $KHCO_3$, 35 m*M* Tris, 35 m*M* HEPES, 40 m*M* potassium gluconate, and 75 m*M* mannitol, and gassed with 95% N_2 and 5% CO_2) or without HCO_3^- (67 m*M* Tris, 73 m*M* HEPES, 55 m*M* potassium gluconate, and 75 m*M* mannitol, and 100% N_2 gassing). Uptake of 10 m*M* Cl^- was determined either in the absence of all gradients (uptake solution: same as preincubation solution without HCO_3^-), in the presence of HCO_3^- alone (uptake solution; 0.285 m*M* $KHCO_3$, 35 m*M* Tris, 35 m*M* HEPES, 85 m*M* potassium gluconate, and 116 m*M* mannitol [pH 7.5]), with or without 5 m*M* DIDS or pH gradient alone (uptake solution: 18 m*M* HEPES, 90 m*M* morpholino-ethane-sulfonic acid (MES), 75 m*M* potassium gluconate, and 98 m*M* mannitol [pH 5.2]), or with both pH and HCO_3^- gradients (uptake solution: 0.285 m*M* $KHCO_3$, 11 m*M* HEPES, 58 m*M* MES, 85 m*M* potassium gluconate, 115 m*M* mannitol, and 2.1 m*M* gluconic acid lactone [pH 5.2] or in the presence of Na^+ and K^+ gradients 50 m*M* Na gluconate, 50 m*M* potassium gluconate, and 35 m*M* Tris [pH 7.5]).

ted onto the middle of a prewetted nitrocellulose filter (0.65-μ pore size; Sartorius Filters, Hayward, CA) and kept under suction. The filter was then immediately washed with 5 ml of ice-cold stop solution and then dissolved in a liquid scintillant. The radioactivity remaining on the filters was counted using a Beckman scintillation counter. Radioactivity remaining on the filters after the incubation media were pipetted onto the radioactive substrates in the absence of vesicles was considered as background and was considered in the calculation. All measurements were performed in triplicate and each experiment was repeated at least three times with freshly prepared membranes. Absolute solute uptake was expressed as nmol/mg of protein.

Calculation and Statistical Analysis. Kinetics curves were fitted using a computerized curve fitting program (Enzfitter, Robin J. Leatherbarrow; Elsevier-Biosoft, Cambridge, UK). Statistical significance was determined using Student's unpaired *t* test or analysis of variance when multiple comparisons were made.

Results

Validation Studies. For marker enzyme studies, the specific activity of Na^+-K^+-ATPase, a marker of basolateral membranes, was enriched 12 ± 2 times as compared with mucosal homogenate. The specific activities of leucine aminopeptidase (brush border membrane marker), NADPH-cytochrome *c* reductase (endoplasmic reticulum marker), and cytochrome *c* oxidase (mitochondrial marker) were impoverished compared with mucosal homogenate by 0.8 ± 0.2-fold, 0.6 ± 0.1-fold, and 0.7 ± 0.1-fold, respectively.

D-Glucose transport showed no overshoot phenomena or sodium dependency, whereas Ca^{2+} transport was markedly enhanced in the presence of ATP (10, 14).

Effect of Extravesicular Osmolarity on Chloride Uptake. To determine how much of the measured uptake represented transport into an intravesicular space, rather than mere nonspecific membrane binding, the intravesicular space was decreased by increasing the

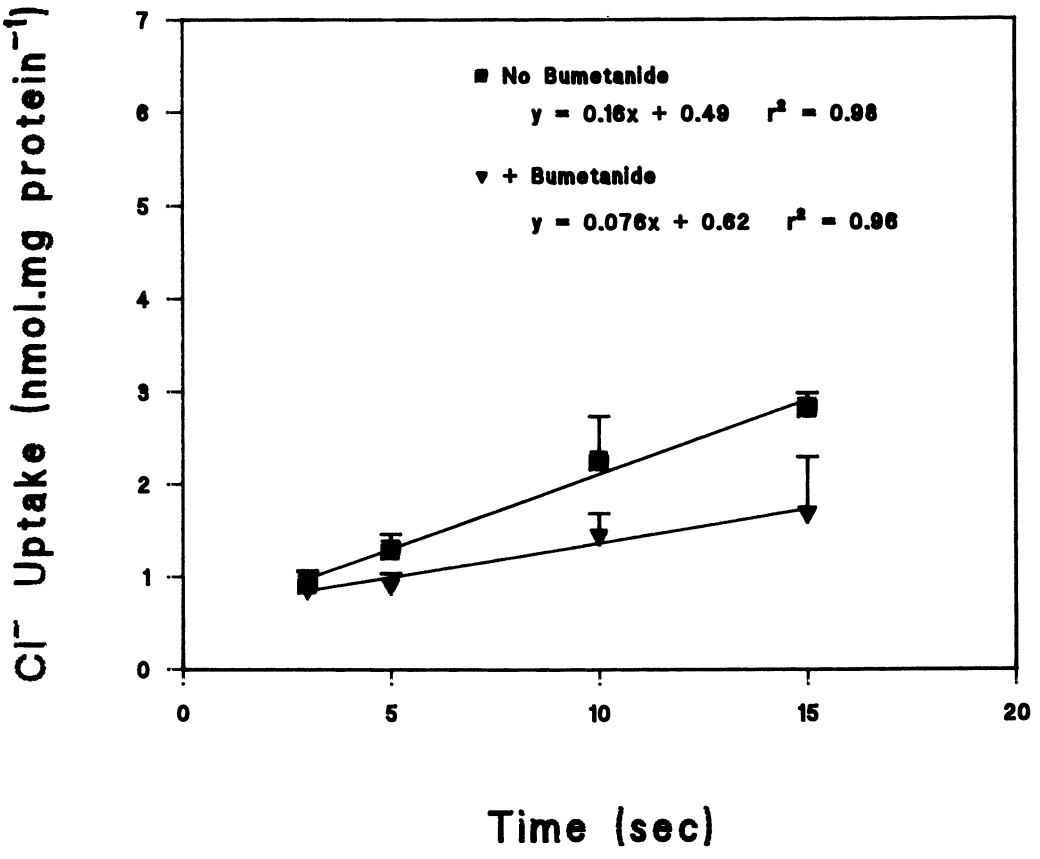

Figure 3. Effect of bumetanide on initial rate of Cl^- uptake. Uptake of Cl^- was determined in the presence of Na^+ and K^+ with and without 0.1 m*M* bumetanide. Cl^- uptake of 10 m*M* Cl^- was determined at 3, 5, 10, and 15 sec.

extravesicular osmolarity with mannitol. Chloride uptake was measured in basolateral membrane vesicles of adult rats at equilibrium in extravesicular osmolarity varying from 250 to 600 mOsm. As shown in Figure 1, chloride uptake varied inversely with extravesicular osmolarity. The relationship between 1/osmolarity and Cl^- uptake was linear $r^2 = 0.99$, with an intercept close to zero, which indicates that Cl^- uptake represents a transmembrane phenomena.

Characterization of Chloride Uptake. The Cl^--HCO_3^- (OH^-) exchange model would predict stimulation of Cl^- uptake by an inwardly directed H^+ gradient and by an outwardly directed HCO_3^- gradient. When Cl^- uptake was compared in the absence of any gradient to uptake under pH gradient, HCO_3^- gradient, or the combination of pH and HCO_3^- gradients, no statistically significant difference was noted (Fig. 2). Moreover, 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS), the anion exchange inhibitor disulfonic stilbene at 5 m*M* concentration (15, 16), did not inhibit Cl^- uptake under a pH gradient condition, thereby indicating the absence of an anion (Cl^-/OH^-) exchange process (Fig. 2). Cl^- uptake values were similar under outwardly directed Na^+ and K^+ with HCO_3^- gradients (2.1 ± 0.2 and 2.04 ± 0.2 nmol/mg protein/30 sec, respectively). This finding indicates that outwardly directed Na^+ and HCO_3^- gradients failed to stimulate Cl^- uptake. The presence of combined Na^+ and K^+ gradients stimulated Cl^- uptake compared with all other imposed gradients (Fig. 2). The time course was followed up to 120 min where equilibrium was obtained. Equilibrium values for all gradients were 1.4 ± 0.2 nmol/mg protein. Peak uptake for Na^+ and K^+ gradient combined was at 15 sec and then declined gradually to equilibrium values. To confirm the presence of $Na^+/K^+/2$ Cl^- process, Cl^- uptake was determined in the presence of Na^+ and K^+ gradients with or without 0.1 m*M* bumetanide. The slope of initial rate of Cl^- uptake was significantly lower in the presence of bumetanide ($P < 0.01$) (Fig. 3). Anthracene-9 carboxylic acid (0.5 m*M*) and diphenylamine-2-carboxylate (0.5 m*M*) agents known to block Cl^- channels (17) had no effect on initial rate of Cl^- uptake (1.9 ± 0.2, 2.0 ± 0.2, and 2.1 ± 0.2 nmol/mg protein/30 sec) for Cl^- channel inhibitors and control, respectively.

Kinetic of Chloride Uptake. The kinetics of bumetanide-sensitive uptake of Cl^- in the presence of Na^+ and K^+ are shown in Figure 3. Uptake was carried out at 5 sec by varying the concentration of Cl^- from 5 m*M* to 55 m*M*. As seen in Figure 4, the relationship between bumetanide-sensitive Cl^- uptake and Cl^- concentration followed Michaelis-Menten kinetics with a V_{max} of 5.6

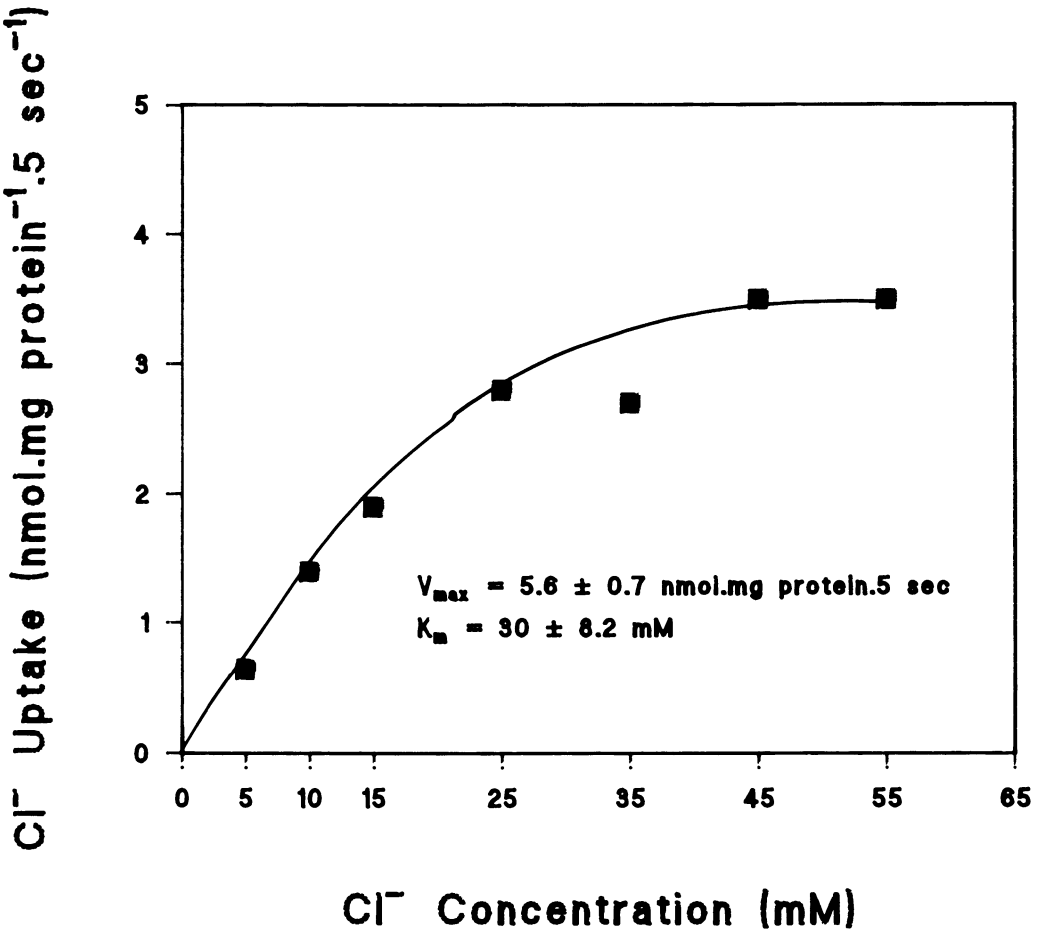

Figure 4. Kinetics of bumetanide-sensitive Cl^- uptake. Cl^- uptake was determined with various concentrations of chloride (5–55 m*M*) in the presence and absence of 0.1 m*M* bumetanide with Na^+ and K^+ in the incubation media. Kinetic parameters were analyzed utilizing Michaelis-Menten kinetics. Each point represents the difference between mean values of six determinations.

± 0.7 nmol/mg protein/5 sec and a K_m of 30 ± 8.2 m*M*.

Effect of Membrane Potential. The effects of ionophore-induced membrane potential on Cl^- uptake were examined using both valinomycin and FCCP. Uptake in the absence of membrane potential (voltage clamp condition) was determined by measuring transport in the presence of valinomycin and with equal concentrations of K^+ present on both sides of the vesicle. Cl^- uptake was significantly greater under negative membrane potential compared with voltage clamp condition ($P < 0.01$) (Fig. 5). To validate the findings with valinomycin, the proton ionophone FCCP was used (18, 19). Cl^- uptake was studied with and without the addition of FCCP. As shown in Figure 5, there was again a statistically significant increase in Cl^- uptake at 15 and 30 sec with positive membrane potential ($P < 0.01$) whether valinomycin-K^+ or FCCP-H^+ was used to generate membrane potential.

Discussion

Studies in our laboratory (15) and by others (20) suggested the presence of Cl^-/HCO_3^- exchange mechanism at the brush border membranes of rat enterocytes. A minor conductive pathway was also present (15). The process by which Cl^- is transported across the basolateral membranes is not well defined.

The present studies did not show any significant stimulation of Cl^- uptake, with outwardly directed pH and HCO_3^- gradients compared with no gradient conditions suggesting the absence of Cl^-/HCO_3^- or Cl^-/OH^- exchange mechanism at the basolateral membranes of rat ileal enterocytes. DIDS, a known inhibitor of anion exchange processes, did not inhibit Cl^- uptake, again indicating the absence of Cl^-/OH^- transport. A similar conclusion was reached by Knickelbein *et al.* (21) in rabbit ileal basolateral membranes. The interaction of Cl^- and HCO_3^- at the renal proximal tubule of the rabbit was investigated by Saski and Yoshiyama (16). Na^+-coupled Cl^-/HCO_3^- and Cl^-/HCO_3^- cotransport processes were demonstrated. Both processes contributed to the overall basolateral HCO_3^- and Cl^- transport. Similar mechanisms of $Na^+/HCO_3^-/Cl^-$ exchange have been demonstrated in basolateral membranes of Necturus proximal tubule (22). The current study dem-

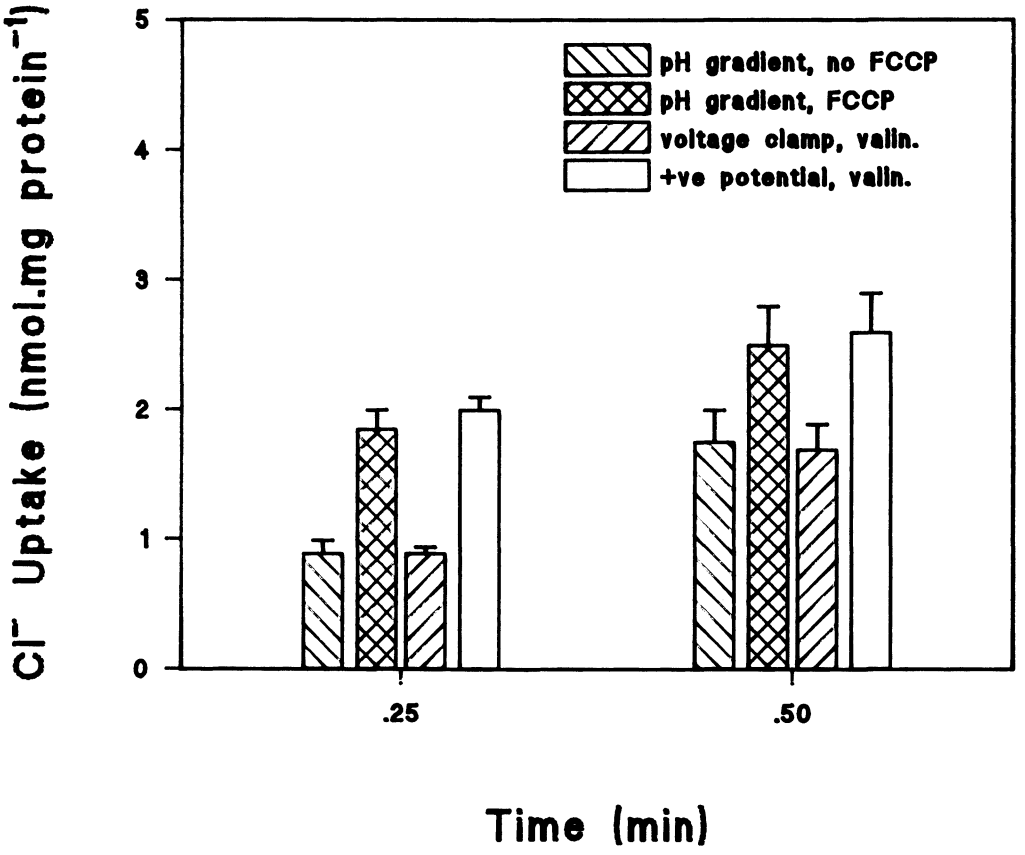

Figure 5. Effect of membrane potential induced by valinomycin and FCCP on Cl^- uptake. Vesicles were preincubated either in the presence of K^+ (70 m*M* HEPES, 70 m*M* Tris, and 100 m*M* K^+ gluconate [pH 7.5]) or absence of K^+ (70 m*M* HEPES, 70 m*M* Tris, and 100 m*M* tetramethylammonium gluconate [pH 7.5]). The uptake of 10 m*M* Cl^- was determined in an uptake solution containing 10 μg of valinomycin/mg protein, 70 m*M* HEPES, 70 m*M* Tris, and 100 m*M* potassium gluconate (pH 7.5). For the FCCP study, vesicles were preincubated with 67 m*M* Tris, 73 m*M* HEPES, 55 m*M* potassium gluconate, and 75 m*M* mannitol (pH 7.5). The uptake of 10 m*M* Cl^- was determined in uptake solution containing 18 m*M* HEPES, 90 m*M* morpholino-ethane-sulfonic acid, 75 m*M* potassium gluconate, and 98 m*M* mannitol (pH 5.2) with or without 80 μ*M* FCCP.

onstrated the absence of the Cl^-/Na^+-HCO_3^- exchange system, which appears to be the predominant process for bicarbonate exit across the basolateral membrane of the rabbit renal proximal tubule (23). Cl^- uptake was driven by a positive membrane potential, as evident by the valinomycin and FCCP studies. The possibility of a negatively charged Cl^- carrier that can be driven by membrane potential cannot be excluded. The relationship between HCO_3^- and Cl^- transport in the rabbit ileum was investigated by Sellin and Desoignie (24). These investigators showed that HCO_3^- secretion is inhibited by serosal Cl^- and bumetanide and stimulated by luminal bicarbonate. The current studies demonstrate the presence of the $Na^+/K^+/2Cl^-$ process at the basolateral membrane, which is inhibitable by bumetanide. This process exhibits saturation kinetics, with V_{max} of 5.6 nmol/mg protein/5 sec and a K_m of 30 ± 8.2 m*M*.

The presence of Cl^- channels at the basolateral membranes was investigated by measuring Cl^- uptake in the presence and absence of the Cl^- channel inhibitors anthracene-9 carboxylic acid and diphenylamine-2-carboxylate. Both agents failed to inhibit Cl^- uptake by basolateral membranes, thereby implying the absence of Cl^- channels.

In summary, the data presented demonstrate that the ileal basolateral membranes of adult rats have a $Na^+/K^+/2Cl^-$ transport process as well as a conductive pathway along a favorable membrane potential gradient. There is no evidence in rat ileum for $Na^+/HCO_3^-/Cl^-$ or Cl^-/HCO_3^-, as previously investigated in rabbit ileum by Sellin and Desoigne (24).

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